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# EXPRESSION AND FUNCTION OF Kv7.4 CHANNELS IN RAT CARDIAC MITOCHONDRIA: POSSIBLE TARGETS FOR CARDIOPROTECTION

**[1]Lara Testai*, [2,3]Vincenzo Barrese*, [4]Maria Virginia Soldovieri, [4]Paolo Ambrosino, [1]Alma Martelli, [4]Iolanda Vinciguerra, [2]Francesco Miceli, [3]Iain Greenwood, [5]Michael J. Curtis, [1]Maria Cristina Breschi, [2]Maria Jose Sisalli, [2]Antonella Scorziello, [5]Miren Josune Canduela, [5]Pedro Grandes, [1]Vincenzo Calderone [#], and [2,4]Maurizio Taglialatela [#]**

**[1]Department of Pharmacy, University of Pisa (Italy)**
**[2]Department of Neuroscience, University of Naples "Federico II", Naples (Italy)**
**[3]Vascular Biology Research Centre, Institute of Cardiovascular and Cell Sciences, St George's, University of London, London (UK)**
**[4]Department of Medicine and Health Science, University of Molise, Campobasso (Italy)**
**[5]Cardiovascular Division, Faculty of Life Sciences & Medicine, King's College London, London (UK)**
**[6]Department of Neurosciences, University of the Basque Country, Leioa (Spain)**

***These Authors contributed equally**
**[#]These Authors contributed equally**

**Corresponding Author:** **Maurizio Taglialatela, MD PhD**
**Department of Medicine and Health Sciences**
**University of Molise**
**Via De Sanctis, 86100 – Campobasso, ITALY**
**Tel. (+39) 0874-404851; Fax: (+39) 0874-404778**
**Email: m.taglialatela@unimol.it**

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# EXPRESSION AND FUNCTION OF Kv7.4 CHANNELS IN RAT CARDIAC MITOCHONDRIA: POSSIBLE TARGETS FOR CARDIOPROTECTION

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## ABSTRACT

**Aims.** Plasmalemmal Kv7.1 (KCNQ1) channels are critical players in cardiac excitability; however, little is known on the functional role of additional Kv7 family members (Kv7.2-5) in cardiac cells. In this work, the expression, function, cellular and subcellular localization, and potential cardioprotective role against anoxic-ischemic cardiac injury of Kv7.4 channels have been investigated.

**Methods and Results.** Expression of Kv7.1 and Kv7.4 transcripts was found in rat heart tissue by qPCR. Western-blots detected Kv7.4 subunits in mitochondria from Kv7.4-transfected cells, H9c2 cardiomyoblasts, freshly-isolated adult cardiomyocytes, and whole hearts. Immunofluorescence experiments revealed that Kv7.4 subunits co-localized with mitochondrial markers in cardiac cells, with about 30-40% of cardiac mitochondria being labelled by Kv7.4 antibodies, a result also confirmed by immuno-gold electron microscopy experiments. In isolated cardiac (but not liver) mitochondria, retigabine (1-30 μM) and flupirtine (30 μM), two selective Kv7 activators, increased $Tl^+$ influx, depolarized the membrane potential, and inhibited calcium uptake; all these effects were antagonized by the Kv7 blocker XE991. In intact H9c2 cells, reducing Kv7.4 expression by RNA interference blunted retigabine-induced mitochondrial membrane depolarization; in these cells, retigabine decreased mitochondrial $Ca^{2+}$ levels and increased radical oxygen species production, both effects prevented by XE991. Finally, retigabine reduced cellular damage in H9c2 cells exposed to anoxia/reoxygenation, and largely prevented the functional and morphological changes triggered by global ischemia/reperfusion (I/R) in Langendorff-perfused rat hearts.

**Conclusions.** Kv7.4 channels are present and functional in cardiac mitochondria; their activation exerts a significant cardioprotective role, making them potential therapeutic targets against I/R-induced cardiac injury.

## 1. INTRODUCTION

Activation of potassium ($K^+$) fluxes across the inner mitochondrial membrane (IMM) is a key mechanism for cardiac ischemic pre-conditioning.[1] In physiological conditions, the IMM is poorly permeable to $K^+$ ions as mitochondrial $K^+$ (mitoK) channels are mostly closed. However, during ischemia, different triggers activate mitoK channels, leading to significant influx of $K^+$ ions, accompanied by water and anions, resulting in matrix swelling and depolarization of the membrane potential. As such, the activation of mitoK channels controls the matrix volume, preserving a narrow intermembrane space, necessary for an effective oxidative phosphorylation, and opposes $Ca^{2+}$-overload and subsequent opening of the mitochondrial permeability transition pore, a powerful trigger of apoptosis.[1] In cardiac cells, pharmacological activation of mitochondrial ATP-sensitive ($mitoK_{ATP}$) and large-conductance $Ca^{2+}$-activated ($mitoBK_{Ca}$) $K^+$ channels[2-4] triggers cardioprotective responses in different models of ischemia/reperfusion (I/R) injury.[3,5-7] Other $K^+$ channel subtypes have been described in the IMM of mostly non cardiac cells.[8] Despite these studies, molecular identification of cardiac mitoK channels heterogeneity is far from being complete.

Voltage-gated $K^+$ channels encoded by the Kv7 gene family (Kv7.1-7.5, also known as KCNQ1-5) have well defined expression pattern and functional roles in heart, neurons, epithelia and vascular and non-vascular smooth muscle.[9] In brain and sensory neurons, Kv7.2/7.3 or 7.3/7.5 heteromers contribute to a sub-threshold current called M-current, whereas Kv7.4 and/or Kv7.5 channels, together with Kv7.1, appear to be major determinants of cellular excitability in vascular and non-vascular smooth muscle cells.[10] In cardiac cells, Kv7.1 encodes for subunits contributing to the slowly repolarizing current $IK_s$;[11] however, significant levels of Kv7.4 transcripts have also been reported in the heart[12], but no function ascribed.

To date all functional roles of Kv7 channels have been attributed to their plasma membrane location and regulations of cellular membrane potential, while their possible impact on mitochondrial physiology has not been determined. In this study, we report biochemical and morphological evidence for the presence of Kv7.4 subunits in cardiac mitochondria, also defining their contribution to mitochondrial function. In addition, we show that the pharmacological activation of these channels exerts significant cytoprotective effects in mitochondrial-dependent in-vitro and ex-vivo models of cardiac I/R damage, suggesting that targeting mitoKv7.4 channels might be an effective strategy for cardioprotection against I/R cardiac injury.

## 2. METHODS

Only a general overview of key methods is provided here; please refer to the Online supplement for further experimental details.

*1. Animals*

Male rats of 2-3 months of age were used. Experimental procedures were carried out following the guidelines of the Directive 2010/63/EU of the European Parliament, and have been approved by the Committee for animal experimentation of the Institutions where experiments were carried out.

*2. RNA extraction and quantitative real-time PCR*

Total RNA from rat brains, livers, and hearts was isolated and reverse transcribed to cDNA; qPCR was carried out in a real-time PCR system using the SYBR Green detection technique and specific primers (Supplementary Table 1).

*3. Isolation of rat primary cardiomyocytes*

Dissected rat hearts were perfused in the Langendorff mode with collagenase. Isolated cells were resuspended in $Ca^{2+}$-Tyrode solution, spread on laminin-coated coverslips and allowed to adhere for 8 hours before further processing.

*4. Cell cultures and transfection*

H9c2 cells from embryonic rat ventricular myocytes, and Chinese Hamster Ovary (CHO) cells were used in the present experiments; cells were transfected using Lipofectamine.

*5. Immunofluorescence*

H9c2 cells or primary cardiomyocytes were incubated with Mitotracker® Red, fixed with paraformaldehyde, and then incubated with primary antibodies. Rat cardiac slices were fixed in paraformaldehyde, and incubated in sucrose for cryopreservation at −80°C; frozen sections (20 µm) were cut and stored at −20°C until further processing. Following incubation with primary and secondary antibodies, cells and cardiac slices were counterstained with Hoechst 33258 to visualize cells nuclei, and analyzed using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope.

*6 Pre-embedding immunogold electron microscopy*

Rat heart coronal vibrosections were incubated with the primary Kv7.4 antibody, and then with a 1.4 nm gold-labeled antibody directed toward mouse IgG. Ultrathin sections containing silver-intensified gold

particles were osmicated, dehydrated, embedded in Epon resin 812, and examined in a PHILIPS EM208S electron microscope.

*7. Mitochondria isolation*

Rat cardiac and hepatic mitochondria were isolated by differential centrifugation;[13] mitochondria from H9c2 cells and primary adult rat cardiomyocytes were obtained using a commercially-available kit. Mitoplasts were prepared using the detergent digitonin.

*8. Western blots*

Protein samples for Western-blot experiments were loaded on 8% or 8-15% SDS–PAGE, and then transferred to a polyvinylidene fluoride membrane. Membranes were incubated with primary and secondary antibodies, and reactive bands detected by chemiluminescence.

*9. Thallium fluxes, membrane potential, and $Ca^{2+}$ measurements in isolated mitochondria from H2c9 cells*

A fluorescent $Tl^+$-sensitive probe (benzothiazole coumarinacetoxy methyl ester; $\lambda$ex = 488 nm, $\lambda$em = 525 nm) was used to evaluate fluxes of the $K^+$-mimetic cation thallium ($Tl^+$) in isolated mitochondria[14] using in a multiplate reader. Mitochondrial membrane potential ($\Delta\psi$) was measured potentiometrically[13] with tetraphenylphosphonium chloride-sensitive mini-electrodes. Mitochondrial $Ca^{2+}$-uptake[13] was evaluated by measuring the changes of the extra-mitochondrial $Ca^{2+}$ concentration using a $Ca^{2+}$ selective mini-electrode.

*10. Membrane potential, calcium concentration ($[Ca^{2+}]_m$), and ROS production monitoring in H9c2 cells mitochondria*

membrane potential, calcium concentration ($[Ca^{2+}]_m$), and ROS production in living H9c2 cells mitochondria were assessed using the fluorescent dyes tetramethyl rhodamine ethyl ester (TMRE), X-Rhod, and MitoSOX Red, respectively. Images from TMRE and X-Rhod experiments were recorded by a conventional immunofluorescence imaging system; those from MitoSOX red, were acquired using confocal microscopy.

*11. Silencing Kv7.4 expression by shRNA in H9c2 cells*

H9c2 cells were transiently transfected with a plasmid encoding for a short hairpin RNA (shRNA) directed against Kv7.4 mRNA (pLKO.1-shKv7.4), or with a control plasmid carrying a nonsense sequence (pLKO.1-scramble). The procedure has been previously used in our lab to selectively suppress Kv7.4 expression in C2C12 rat skeletal myoblasts; Kv7.4 silencing was assessed by western-blotting experiments.[15]

*16. Anoxia/reoxygenation in H9c2 cells*

To simulate anoxia in H9c2 cells, plated cells were sealed for 16 hours in airtight containers saturated with 95% N2 and 5% CO2 (37°C), while twin plates were placed in 95% air and 5% CO2 (normoxic conditions). Then, cells were subjected to reoxygenation for 2 hours in an atmosphere containing 95 % air and 5% CO2 (37°C). After reoxygenation, cell viability was assessed.

*17. Langendorff-perfused rat hearts*

Rat hearts were quickly removed, mounted on a Langendorff apparatus, and perfused with oxygenated solution at 37 °C and constant pressure (70-80 mmHg).[16] A water-filled latex balloon connected to a pressure transducer was introduced into the left ventricle to achieve a stable left ventricular end-diastolic pressure of 5-10 mmHg. The functional parameters of heart rate (HR), left ventricular developed pressure (LVDP) and the rate of rise of the left ventricular pressure (dP/dt) were monitored continuously. Rate pressure product (RPP) was calculated as HR x LVDP. Coronary flow (CF) was also estimated. After 20 min of equilibration, 30 min of global ischemia (no flow) followed. At the end of the ischemic period, the hearts were reperfused for 2 hours; following reperfusion, hearts were removed from the Langendorff apparatus, and 2mm large slices cut from the left ventricle were immersed in a solution containing 2,3,5-triphenyltetrazolium chloride to evaluate the extension of the damage.

*18. Drugs and antibodies*

Retigabine and flupirtine were from Valeant (Laval, Canada); XE991, olygomicin, 2,4-dinitrophenol, valinomycin, and FCCP were from Sigma-Aldrich (Milan, Italy). The primary antibodies used were: i) mouse monoclonal Kv7.4 (UC Davis/NIH NeuroMab Facility, USA; clone N43/6.1; dilution: 1:100 for immunofluorescence and electron microscopy; 1:500/1000 for western blot); ii) rabbit polyclonal Sphingosine 1-phosphate receptor 1 (S1PR1, 1:500; Abcam, Cambridge, UK); iii) mouse monoclonal Kv7.1 (1:500; NeuroMab, clone N37A/10.1, used for western blot); iv) rabbit Kv7.1 (1:100; Millipore, Temecula, USA, used for immunocytochemistry); v) mouse VDAC1 (TC supernatant, 1:5, NeuroMab); vi) mouse α-tubulin antibody (1:5000; Sigma-Aldrich); vi) mouse GAPDH (1:5000; Sigma-Aldrich); viii) rabbit COX-IV (Abcam, Cambridge, UK). Secondary antibodies for immunofluorescence (Jackson ImmunoResearch, Newmarket, UK) were: i) anti-mouse conjugated to Alexa Fluor 488; ii) anti-rabbit conjugated to Alexa Fluor 488; iii) anti-rabbit conjugated to Alexa Fluor 568. Anti-mouse or anti-rabbit conjugated to HRP secondary antibodies (Jackson ImmunoResearch, Newmarket, UK) were used for Western blot experiment, at 1:5000 dilution.

*19. Statistics*

Data, reported as Mean±SEM, were statistically analysed by ANOVA followed (if F reached significance) by the Tukey test (true or not, Vincenzo? Or did you use another one?), or by the Student's t test (software: GraphPadPrism 4.0). P values < 0.05 were considered as indicative of significant differences.

## 3. RESULTS

### 3.1 Expression of Kv7.4 mRNA and protein in cardiac tissue and cardiomyocytes

Quantitative PCR experiments using specific primers (Supplementary Table 1) were performed on mRNA extracted from rat brain, heart and liver. In the brain, Kv7.2-5 transcripts were readily identified, whereas smaller but detectable amounts of Kv7.1 transcripts were also found (Fig. 1A). By contrast, mRNAs encoding for Kv7.2-5 were expressed at rather marginal levels in the liver, whereas Kv7.1 transcripts were clearly detected. In cardiac tissue, Kv7.1 showed the highest expression level among Kv7 transcripts, but Kv7.4 transcripts were also found,[12] with expression levels comparable to the brain.

Western blots experiments with anti-Kv7.4 antibodies in total lysates from rat heart lysates revealed a band of ~77kDa; this band was enriched in voltage-dependent anion-selective (VDAC) channel-enriched mitochondrial fractions, which tested negative for cytosolic markers such as $\alpha$-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 1B). Kv7.4 signals were also readily detected in rat heart mitoplasts, in which the outer mitochondrial membrane (OMM) VDAC signal was markedly decreased (Fig. 1C). A band of similar molecular mass was also observed in Kv7.4-transfected CHO cells, but not in CHO cells transfected with Kv7.1, Kv7.2, Kv7.3 or Kv7.5 cDNAs, or in non-transfected CHO cells (Suppl. Fig. 1A), confirming antibody specificity. Also in Kv7.4-transfected CHO cells, this band was highly enriched in mitochondrial fractions (Suppl. Fig. 1).

In H9c2 cardiomyoblasts, Kv7.4 antibodies identified a 77kDa band in mitochondria (positive to cytochrome c oxidase subunit IV, COX-IV), but not in cytoplasmic (tubulin-positive) or microsomal (tubulin-negative) fractions (Fig. 1D); a similar subcellular pattern of expression of Kv7.4 subunits was also observed in freshly isolated rat cardiac myocytes, where the Kv7.4-reactive band was mainly found in COX-IV-positive, but GADPH- and PDI-negative fractions corresponding to mitochondria. By contrast, in cardiac myocytes, a 71 kDa band corresponding to Kv7.1 subunits could be readily detected in the cytosolic fraction, but was absent from the microsomal and mitochondrial fractions (Fig. 1E). A band of similar molecular mass was revealed in total lysates from Kv7.1-transfected (but not Kv7.2-, Kv7.3-, Kv7.4-, or Kv7.5-transfected) CHO cells (Suppl. Fig. 1B); this band was not detected in mitochondrial preparations from Kv7.1-transfected cells (Suppl. Fig. 1C).

### 3.2 Subcellular localization of Kv7.4 in H9c2 cells, isolated primary rat cardiomyocytes, and adult rat heart

In H9c2 rat embryonic cardiomyocytes, immunofluorescence experiments revealed that Kv7.4 antibodies stained a network of filamentous structures surrounding the nucleus and spreading throughout the cytosol (Fig. 2A, panels a and d); the same subcellular structures were also labelled by the mitochondria-specific marker Mitotracker (Fig. 2A, panel b; merge image in Fig. 2A, panel c). Better resolution was obtained in higher magnification images (Fig. 2A, panels d-f). By contrast, H2c9 cell staining

with antibodies directed against type-1 Sphingosine-1-phosphate receptor (S1PR1)[17] clearly labelled the plasma membrane (indicated by the arrow heads) and the cytoplasm (Fig. 2AB, panel h), and did not co-localize with that of Mitotracker (data not shown) or of Kv7.4 antibodies (Fig. 2A, panel g). Notably, no Kv7.4-specific signal was detected in Mitotracker-labelled mitochondria of non-transfected CHO cells (Suppl. Fig. 2), arguing against an unspecific targeting of anti-Kv7.4 antibodies to mitochondria.

Acutely-isolated rat adult primary cardiomyocytes displayed typical rod-like shape and rectangular ends (Figs. 2B, panels a-l).[18] In these cells, Mitotracker (red pseudocolor) stained longitudinal arrays of mitochondria running in parallel with the sarcoplasmic reticulum[19] and clustering around the cell nuclei[16] (arrows in Figs. 2B, panels a, d, g, and j). The same structures were also labelled by Kv7.4 antibodies (Figs. 2B, panels b and e), as shown in the merge panels (c and f, at lower and higher magnification, respectively); the Pearson's coefficient for Kv7.4 and mitotracker co-localization was 0.33±0.01 (n=???), suggesting that not all mitochondria express a detectable amount of Kv7.4 subunits. Conversely, staining produced by Kv7.1 antibodies was transversely-oriented (Figs. 2B, panels h and k) and did not colocalize with Mitotracker (Fig 2B, panels g and j; merge panels in I and l); the Pearson's coefficient for Kv7.1 and mitotracker co-localization was 0.02±0.02 (n=???).

In rat heart slices, Kv7.4 antibody staining displayed the same longitudinal pattern observed in isolated cardiomyocytes, with positively-labeled structures running along the major axis of cardiac fibers and surrounding the contractile myofilaments (Fig. 2B, panel n). The expression pattern of Kv7.4 subunits overlapped considerably with that of mitochondrial COX-IV (Fig2B, panels m and o).

### 3.2 Subcellular localization of Kv7.4 subunits in adult rat heart detected by immunogold electron microscopy

To confirm the localization of Kv7.4 to mitochondria, pre-embedding immunogold electron microscopy experiments were performed in rat adult heart tissue using Kv7.4 antibodies. The upper part of Fig. 3 shows four representative images from 3 different hearts where gold labeled particles were detected in the mitochondria, as defined by their distinctive cristae. In 138 images analyzed, 41.2% of the mitochondria were labeled by gold particles (Fig. 3, lower left panel), a value consistent with immunofluorescence analysis results. In most cases (66.7%, corresponding to 112 mitochondria), labeling was associated to mitochondrial membranes (periphery or cristae), whereas in 22.6% (38 mitochondria) was predominantly located in the mitochondrial matrix; 10.7% (18 mitochondria) were labelled both at the membrane and intramitochondrial level (Fig. 3, lower right panel).

### 3.3 Effects of Kv7 activators on $Tl^+$ fluxes, membrane potential and $Ca^{2+}$ uptake in isolated rat heart mitochondria

Isolated rat heart mitochondria exposed to the $K^+$ ionophore valinomycin (2 μM) showed a marked increase in their trans-membrane permeability to $Tl^+$. Increasing concentrations of the Kv7.2-7.5-activators retigabine (1-30 μM) and flupirtine (30 μM) also promoted $Tl^+$ influx; the $pEC_{50}$ for retigabine was 5.59±0.05 (Fig. 4A). The initial rate of fluorescence increase induced by valinomycin was 336±70 ΔF/sec; those for 1, 3, 10 and 30μM retigabine were 19±37, 134±33, 151±43, and 211±41 ΔF/sec, respectively. In heart mitochondria, the effects of retigabine and flupirtine were abrogated by the selective Kv7-blocker XE991 (10 μM), which had no effect on valinomycin-induced $Tl^+$ influx (Fig. 5B). Instead, in mitochondria isolated from rat adult hepatic tissue (where Kv7.4 mRNA levels are virtually undetectable; Fig. 1A), retigabine was ineffective in triggering $Tl^+$ influx, whereas valinomycin was still effective (Fig. 4A).

In cardiac, but not in liver, mitochondria retigabine also produced an XE991 (10 μM)-sensitive concentration-dependent depolarization of the mitochondrial membrane, with a $pEC_{50}$ of 5.19±0.08 (Fig. 4C).

Incubation of isolated heart and liver mitochondria in a $Ca^{2+}$-rich solution (100 μM) caused a rapid and almost complete uptake of the cation into the mitochondrial matrix, reducing its extracellular free concentration. In heart mitochondria, this effect was significantly reduced by retigabine (30 μM); XE991 (10 μM) did not influence resting mitochondrial $Ca^{2+}$ uptake, but completely antagonized the effects of retigabine. In contrast, retigabine did not influence $Ca^{2+}$ uptake in liver mitochondria (Fig. 4D).

### 3.4 Effects of retigabine on mitochondrial membrane potential, $Ca^{2+}$ levels, and ROS production in H9c2 cardiomyoblasts

To confirm that Kv7.4 activation could influence mitochondrial function in intact cardiac cells, mitochondrial membrane potential, $Ca^{2+}$ levels, and radical oxygen species (ROS) production were measured in intact H9c2 rat cardiomyoblasts. Exposure of H9c2 cells to 30 μM retigabine irreversibly decreased TMRE fluorescence intensity, indicative of mitochondrial depolarization (Figs. 5A and 5C), with an efficacy about 20% of that of the mitochondrial uncoupler FCCP (1 μM). Retigabine $IC_{50}$ was 13.1±1.1 μM (n=6-11). XE991 (10 μM) did not modify TMRE fluorescence, but largely abolished RET-induced inhibition of TMRE fluorescence (Figs. 5B and 5C).

To assess the specific contribution of mitoKv7.4 channels in retigabine-induced effects on mitochondrial membrane potential, H9c2 cells were transfected with a short hairpin RNA targeted against Kv7.4 mRNA (sh-Kv7.4).[15] Western blots experiments confirmed that Kv7.4 expression was reduced to 51.0+6.0% (n=5) in total lysated from shKv7.4-transfected cells (inset in panel 5D); a smaller reduction of the Kv7.4 signal was instead observed (74.3+3.3%; n=5) upon transfection with a control plasmid (scr). In

shKv7.4-trasfected (but not in src-transfected) H9c2 cells, retigabine (30 μM)-induced inhibition of TMRE fluorescence intensity was decreased when compared to un-transfected H9c2 cells (Figs. 5D and 5E).

Exposure of H9c2 cells to 30 μM retigabine reversibly decreased X-RHOD-1 fluorescence intensity, suggesting a decrease in mitochondrial $Ca^{2+}$ levels (Figs. 6A and 6B); an opposite effect was instead promoted by the mitochondrial uncoupler FCCP (1 μM). XE991 (10 μM) prevented retigabine-induced decrease in X-RHOD-1 fluorescence (Fig. 6B). Retigabine (30 μM) also increased mitochondrial ROS production in H9c2 cells; XE991 (10 μM) largely prevented retigabine-induced ROS increase (Fig. 6C).

**3.5 Effects of retigabine on H9c2 cardiomyoblasts survival following anoxia/reoxygenation (A/R)**

H9c2 cardiomyoblasts exposed to 16 hours of anoxia, followed by 2 hours of reoxygenation, exhibited a significant decrease in viability. Preincubation with retigabine (100μM; applied one hour before and throughout the anoxic period, but not in the reoxygenation phase), failed to modify cell viability in normoxic conditions, but significantly increased H9c2 cells survival after the A/R injury. The Kv7 blocker XE991 did not influence the cell viability in both normoxic and A/R conditions, but fully antagonized retigabine-induced protection of cardiomyoblasts during A/R (Fig. 7A). To investigate whether retigabine-induced ROS formation contributed to retigabine-induced cardioprotection, the effect of vitamin E (vitE) was also evaluated. VitE (50 μM) failed to affect cell vitality in normoxic conditions, fully prevented A/R-induced H9c2 cell death, but did not prevent retigabine (100 μM)-induced cytoprotection when incubated 1 hour before and together with the Kv7.4 activator (Supplementary Fig. 3).

**3.6 Cardioprotective effects exerted by Kv7 channel activation in Langendorff-perfused adult rat hearts**

During reperfusion following 30 min of global ischemia, vehicle-treated hearts exhibited a reduction of the inotropic functional parameters. In particular, the rate pressure product (RPP) and the maximal rate of rise of the left ventricular pressure (dP/dt) always remained lower than the corresponding pre-ischemic values (Figs. 7B and 7C, respectively). In vehicle treated hearts, the coronary flow (CF) recorded during the reperfusion time following the ischemic episode was significantly reduced as compared to the pre-ischemic phase (Fig. 7D). Retigabine exposure (100μM, perfused during the pre-ischemic phase only), led to an almost complete recovery of the RPP, dP/dt, and CF during reperfusion (Figs. 7B-D). By contrast, no retigabine-induced cardioprotection was observed when the drug was administered upon reperfusion, after the ischemia/reoxygenation (I/R) cycle (what parameter was measured? Functional or morphological? data not shown? Could we add some data in the text?). Morphometric analysis revealed an almost 50% decrease in tissue vitality in the left ventricles from I/R-treated hearts (expressed as Ai/Alv); treatment with retigabine (100μM) during the pre-ischemic phase led to a significant reduction of the tissue injury (Figs. 7E and 7F). Noteworthy, retigabine (100μM)-induced beneficial effects on functional (Figs. 7B-D) and morphometric (Fig. 7F) parameters was largely abolished by XE991 (10μM) pretreatment.

## 4. DISCUSSION

The fine tuning of the mitochondrial membrane potential is a critical factor in controlling cell fate during physiological or pathological states, such as myocardial I/R injury, and the pharmacological modulation of mitochondrial ion channels appears as an innovative cardioprotective strategy. In this study, we provide the first evidence that $K^+$ channels of the Kv7.4 subclass localize to mitochondria in cardiac myocytes, and that their pharmacological activation depolarizes the mitochondrial membrane potential, reduces mitochondrial $Ca^{2+}$ uptake, and attenuates damage following I/R.

Quantitative PCR experiments revealed that the rat heart expressed Kv7.1 transcripts at high levels, a result consistent with the well described contribution of Kv7.1 subunits to $I_{Ks}$, the late repolarizing current of the cardiac action potential.[11] In addition, as previously suggested in mouse[12] and zebrafish,[21] moderate levels of Kv7.4 transcripts were also observed in the heart, whereas expression levels of Kv7.2, Kv7.3, and Kv7.5 genes were negligible, suggesting that, in addition to their roles in vascular and non-vascular smooth muscles[10] and in the auditory system,[22] Kv7.4 channels may also play a critical role in cardiac physiology.

Western blot experiments confirmed the abundant expression of Kv7.4 subunits in rat cardiac tissue, and revealed that rat heart subcellular fractions highly enriched in mitochondria were intensively positive for Kv7.4 subunits, suggesting their preferential location in mitochondria; experiments in mitoplasts confirmed Kv7.4 subunit expression on the IMM. Rat heart samples used in these experiments likely contain a substantial proportion of vascular tissue, where Kv7.4 channels are known to be expressed;[23,234] therefore, similar experiments were also carried out in H9c2 rat cardiomyoblasts,[25] and in freshly-isolated adult cardiomyocytes. In both cell types, biochemical experiments revealed Kv7.4 subunits mainly in VDAC- or COX-IV-positive mitochondrial fractions.

Immunofluorescence analysis in H9c2 cells confirmed that Kv7.4 expression pattern overlapped that of the mitochondrial marker Mitotracker, and was clearly distinct from a plasma membrane GPCR such as type-1 Sphingosine-1-phosphate receptor (S1PR1).[26] In freshly-isolated cardiomyocytes, Kv7.4 antibodies also labelled Mitotracker-positive longitudinal structures corresponding to mitochondria running in parallel to their major axis. Consistent with previous work,[27] Kv7.1 antibody staining was predominantly transversely-oriented, with striations resembling those of the transverse component of the T-tubular system of adult ventricular myocytes.[28] Similarly, immunohistochemical experiments in adult cardiac slices revealed that Kv7.4 displayed a longitudinally-oriented punctuate staining pattern likely corresponding to single, dot-like mitochondria,[19] similar to that of the mitochondrial marker COX-IV. In the same preparation, electron microscopy confirmed labeling of Kv7.4 subunits in about 40% of the mitochondria, with a preferential location on internal (cristae) or peripheral membranes.

To investigate the functional significance of cardiac mitoKv7.4 channels, Kv7 activators (retigabine and flupirtine) and blockers (XE991) were used. Retigabine and flupirtine act on channels formed by all Kv7 subunits, except Kv7.1.[29,30] In rat heart mitochondria, both retigabine and flupirtine increased $Tl^+$ influx,

with potency values consistent with their ability to enhance Kv7.4 currents in electrophysiological experiments.[31] The effects of Kv7 activators on $Tl^+$ fluxes across heart mitochondrial membranes closely resemble those of the mito$K_{ATP}$-opener diazoxide,[14,32] and of naringenin, a mito$BK_{Ca}$-opener.[16] Noteworthy, XE991 antagonized $Tl^+$ influx triggered by retigabine and flupirtine, but not by valinomycin, confirming a specific involvement of Kv7 channels. Retigabine also evoked concentration-dependent and XE991-sensitive mitochondrial depolarization, a result consistent with the recognized effect of an increased IMM $K^+$ permeability on mitochondrial membrane potential.[4] The extent of retigabine-induced mitochondrial depolarization is similar to that shown by activators of $K_{ATP}$ channels, such as diazoxide, pinacidil[33] and benzopyrane-derived selective mito$K_{ATP}$-openers, as well as by $BK_{Ca}$-openers.[13] Retigabine was also effective in depolarizing the mitochondrial membrane potential in intact H9c2 cells; this effect was blocked by XE991 as well as by reducing Kv7.4 expression with shRNAs, providing genetic evidence for a specific role for Kv7.4 channels in the pharmacological effects herein described.

Mitochondria avidly accumulate $Ca^{2+}$ ions into the matrix, thus buffering excessive increases in free cytosolic $Ca^{2+}$.[34] Both in isolated mitochondria and intact H9c2 cells, retigabine decreased mitochondrial $Ca^{2+}$ uptake in an XE991-sensitive manner, suggesting that even relatively small positive shifts of the mitochondrial potential substantially reduce $Ca^{2+}$ uptake.[13] In mitochondria isolated from hepatic tissue, where Kv7.4 mRNA levels were almost undetectable, retigabine failed to affect $Tl^+$ fluxes, mitochondrial membrane potential, and $Ca^{2+}$ uptake, suggesting that retigabine-evoked effects in cardiac mitochondria are selectively mediated by Kv7.4 channels.

Activation of mitochondrial $K^+$ channels such as mito$K_{ATP}$, mito$SK_{Ca}$ and mito$BK_{Ca}$ promotes protective effects against cardiac ischemic injury.[1] In cultured H9c2 cells exposed to A/R, retigabine attenuated cell injury, and XE-991 antagonized the protective effects of the Kv7 activator. Noteworthy, neither retigabine nor XE991 influenced the viability of H9c2 cells exposed to normoxic environment, thus, suggesting specific anti-ischemic mechanisms of protection involving the activation of Kv7 channels. In H9c2 cells, retigabine increased ROS formation, a result lending support to the hypothesis that an initial $K^+$ entry via mitoKv7.4, by promoting a mild oxidative stress, would prevent opening of the mitochondrial permeability transition pore and decrease anoxic cell death.[35] However, vitamin E did not prevent retigabine-induced H9c2 cardioprotective effects; although this result seems to suggest that retigabine-induced cytoprotection is not directly caused by an increased ROS production, vitamin E likely targets a myriad of molecular steps triggered by anoxia-reoxygenation, hampering a potential inhibition of retigabine-induced cytoprotection.

In Langendorff-perfused rat hearts submitted to I/R, retigabine added during the pre-ischemic phase improved all the functional and morphological parameters of post-ischemic recovery; also these effects were fully abolished by XE991. However, these results need to be interpreted with caution, since sarcolemmal Kv7.4 channels identified in the vascular smooth muscle of rat coronary arteries mediate

significant vasorelaxing actions[36] which may participate in the observed cardioprotective effects. However, retigabine-induced functional and structural protection was assessed after 2 hours of drug-free post-ischemic recovery, suggesting a major contribution of retigabine-sensitive cardiac mitoKv7.4 channels in cardioprotection against I/R. This view seems to be confirmed by the observation that retigabine was ineffective when administered only upon reperfusion, after the I/R cycle; however, further experiments are needed to dissect the relative contribution of Kv7.4 channels in cardiomyocytes and/or vascular smooth muscle cells in cardioprotection triggered by the Kv7.4 activator. The fact that retigabine-induced beneficial effects required drug concentrations higher than those affecting mitochondrial function may reflect a limited drug delivery to the mitochondrial target across the plasma membrane.

Overall, the results obtained demonstrate that rat cardiomyocytes express mitochondrial Kv7.4 channels which, by regulating membrane potential, influence mitochondrial $Ca^{2+}$ permeability. The pharmacological activation of myocardial mitoKv7.4 channel promotes structural and functional recovery following I/R injury, highlighting new and appealing therapeutic strategies for cardioprotection.

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## FIGURE LEGENDS

**Fig 1. Kv7.4 expression in rat heart. A.** Quantitative PCR showing Kv7 mRNA levels in rat brain, heart and liver. Cycle threshold (Ct) are normalized to the housekeeping gene GAPDH, using the $2^{-\Delta Ct}$ formula. Data are from 3 separate experiments. **B.** Western blot for Kv7.4, α-tubulin, GAPDH and VDAC in rat heart mitochondria (mito) and whole homogenate (total). **C.** Western blot for Kv7.4 in rat cardiac mitochondria before (Mito) and after their exposure to digitonin (1X for 15 min, 2X for 15 min, 1X for 45 min). Successful mitoplasts isolation was indicated by the preservation of the IMM marker COX IV and the disappearance of the OMM marker VDAC. **D.** Western blot for Kv7.4, α-tubulin, and COX IV in H9c2 cells mitochondrial (Mito), cytosolic (Cyto) and microsomal (Micr) fractions; molecular weights are on the left. **E.** Western blot for Kv7.4, Kv7.1, GADPH, PDI, and COX IV in mitochondrial (Mito), cytosolic (Cyto) and microsomal (Micr) fractions from freshly-isolated adult rat cardiomyocytes. In all panels, data are representative of 4 experiments and markers molecular weights are shown on the left.

**Fig 2. Subcellular localization of Kv7.4 in rat H9c2 cardiomyoblasts and in primary rat cardiomyocytes. A.** Immunofluorescence in H9c2 cells. Mitochondria labelled with Mitotracker are in red in panels b and e; Kv7.4 antibody labelling is in green (panels a, d, and g); labelling with S1PR1 antibodies is in green (panel h); nuclear Hoechst staining is in blue. Arrows: mitochondria; arrow-heads: plasma membrane. Merged images are in panels c, f, and i. Panel d-e are enlargements of the region boxed in red in panel c. Scale bar, 5µm. **B.** *Panels a-l*: immunofluorescence in acutely-isolated adult rat cardiomyocytes. Mitochondria labelled with Mitotracker (red in panels a, d, g, j), were then stained for Kv7.4 (green, panels b and e) or Kv7.1 (green, panels h and k); nuclei (blue) were counterstained with Hoechst. Merged images are in panels c, f, i, and I. The second and the fourth row set of panels are higher magnifications of images shown in the first and third row, respectively. Scale bar is 10µm (panels c, i) or 5µm (panels f, l). Arrows: mitochondria. *Panels m-o*: immunofluorescence in rat cardiac slices incubated with anti COX-IV (red) and Kv7.4- antibody (green); nuclei were counterstained with Hoechst (blue). Scale bar: 10µm. Experiments was repeated 3 times, with similar results.

**Figure 3. Electron immunogold detection of Kv7.4 subunits in mouse cardiomyocytes mitochondria.** Four representative images (A-D), each from different sections, are shown. Scale bar: 500 nm. Black arrows indicate mitochondria. The lower panels show the quantification of gold particles distribution in 408 mitochondria (138 images analyzed; sections from 3 separate animals). The left panel shows the percentage of gold-labelled ad un-labelled mitochondria; for gold-labelled mitochondria, the right panel reports the particles distribution in the membranes (periphery or cristae), intramitochondrially (inside), or at both locations. Data are expressed as Mean±S.E.M.

**Figure 4. The Kv7 activator retigabine regulates thallium influx, membrane potential, and $Ca^{2+}$ permeability in cardiac mitochondria. A.** Concentration-response curve for retigabine-induced thallium influx into the matrix of heart (HM) or liver (LM) mitochondria. **B.** Effect of valinomycin (Val, 2μM), retigabine (Ret, 30μM), and flupirtine (Flu, 30 μM) on thallium influx into the heart mitochondrial matrix, in the absence or in the presence of XE991 (10μM). Data are expressed as % of the valinomycin-induced response. **C.** Concentration-dependent effect of retigabine on membrane potential of heart mitochondria (HM), in the absence or in the presence of XE991 (10μM); drug effects on rat liver mitochondria (LM) are also reported. **D.** Changes of extramitochondrial $Ca^{2+}$ concentration upon isolated heart (HM, black columns) or liver (LM, gray columns) mitochondria incubation with vehicle (Ctl) or retigabine (Ret, 30μM), in the absence or in the presence of XE991 (10μM). In all panels, each data point is the Mean±SEM of 6 experiments run in triplicate, each from mitochondria from a different rat heart. Asterisks indicate significant statistical differences (*=P<0.05; ***=P<0.001).

**Fig. 5. Effect of Kv7 modulators on mitochondrial membrane potential in control and Kv7.4-silenced H9c2 cells. A,B.** Time-course of TMRE fluorescence intensity measured in single H9c2 cells in control solution (Normal Krebs, NK), Retigabine (30 μM, Ret), FCCP (1 μM) and/or XE991 (10 μM); treatment duration is indicated by the bar on top of the traces. **C.** Quantification of the data reported in **A** and **B**, normalized to fluorescence intensity values measured in control solution. Each bar is the mean±SEM of 6-65 separate determinations, each performed in a single cell, recorded in 5 separate experimental sessions. The asterisks denote values significantly different from controls. **D.** Time-course of the effects of 30 μM Retigabine (Ret) perfusion (started where indicated by the arrow) on TMRE fluorescence intensity measured in H9c2 cells non transfected (NT; gray diamonds) or 72 hours after transfection with scramble (Scr; filled dots) or shKv7.4 (white squares) plasmids. The inset shows a representative Western blot experiment on total lysates of H9c2 cells non-transfected (NT) or 72 hours after transfection with scramble (Scr) or shKv7.4 plasmids. Total lysates from non-transfected CHO cells (NT) or expressing Kv7.4 subunits were also loaded as controls. The numbers and the arrows on the left of the image indicate the position and the molecular mass of the protein markers. **E.** Quantification of the fluorescence intensity measured at steady-state time points (~ after 8 min of Ret perfusion) normalized to controls. Each bar is the mean±SEM of 16-55 separate determinations, each performed in a single cell, recorded in 3 separate experimental sessions (3 separate transfections). The asterisks denote statistically significant differences (p<0.05).

**Fig. 6. Effect of Kv7 modulators on $Ca^{2+}$ and ROS levels in H9c2 cells. A.** Representative trace from a single X-Rhod-1-loaded H9c2 cell in control solution (Normal Krebs, NK) or exposed to retigabine (30 μM; Ret) or FCCP (1 μM), as indicated by the bar on the top of the trace. **B.** Quantification of X-Rhod-1 fuorescence in H9c2 cells exposed to the experimental conditions indicated. Each bar is the mean±SEM of 16-49 separate

determinations, each performed in a single cell, recorded in 3 separate experimental sessions. The asterisks indicate values significantly different from controls ($p<0.05$). **C.** Effect of Retigabine (30 μM; Ret) on MitoSOX fluorescence intensity in H9c2 cells. Each bar is the mean±SEM of 36-59 separate determinations, each performed in a single cell, recorded in 3 separate experimental sessions. The asterisks indicate statistically significant differences ($p<0.05$).

**Figure 7. Cardioprotection by Kv7 modulators in *in vitro* anoxia and *ex-vivo* ischemia models. A.** Viability of H9c2 cardiomyoblasts exposed to normoxic conditions (black columns) or to anoxia/reoxygenation (white columns) treated with: vehicle (Ctl), retigabine (Ret, 100μM), XE991 (XE991, 10μM) and retigabine 100μM plus XE991 10μM (Ret+XE991), expressed as % of vehicle-treated cells exposed to normoxic conditions. Asterisks indicate significant statistical differences, analysed by one-way ANOVA (*=P<0.05; ***=P<0.001). Data are from 6 experiments each performed in triplicate. Time-course of RPP (Rate x Pressure Product; **B**), dP/dt (**C**), and coronary flow (**D**) in Langendorff-perfused hearts treated with vehicle, retigabine (100 μM) or retigabine (100 μM) plus XE991 (10 μM). Data are expressed as % of the respective values recorded in the pre-ischemic phase. In **B**, **C**, and **D**, two-way ANOVA analysis indicated that the vehicle and retigabine curves exhibit highly significant (***=P<0.001) differences. **E.** Representative images of left ventricle slices from vehicle- or retigabine-treated (100 μM) hearts after ischemia/reperfusion. **F.** Quantification of the extension of the ischemic damage (white/pale regions), expressed as % of the left ventricle slice area (Ai/Alv) in the indicated groups. The asterisk indicates a statistically-significant difference (*=P<0.05). Data are from 6-8 experiments each performed in different animals.